 Human Molecular Genetics, 2002, Vol. 11, No. 9 1045–1058

Identification of transcriptional targets for Six5: implication for the pathogenesis of myotonic dystrophy type 1

Shigeru Sato[1], Miwa Nakamura[1], Diane H. Cho[2], Stephen J. Tapscott[2], Hidenori Ozaki[1] and Kiyoshi Kawakami[1,*]

[1]Department of Biology, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan and [2]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109-1024, USA

Received December 19, 2001; Revised and Accepted March 7, 2002

Myotonic dystrophy 1 (DM1) is the most common inherited neuromuscular disease in adults. The disorder, characterized by myotonia, muscle wasting and weakness, cataract, insulin resistance, and mental impairment, is caused by the expansion of an unstable CTG repeat located in the 3′ untranslated region of *DMPK*. The repeat expansion suppresses the expression of the homeobox gene *SIX5*. We describe here an experimental system to identify downstream transcriptional targets of mouse Six5 in order to elucidate the role of *SIX5* in the pathogenesis of DM1 and development. By overexpressing a constitutively active Six5 (VP16–Six5wt) using adenovirus-mediated gene transfer in P19 cells and subsequent expression profiling using cDNA arrays, 21 genes, whose expression level increased by the treatment, were identified as potential target genes. Genes expressed in the somites, skeletal muscles, brain and meninges comprised the majority, suggesting the role of *Six5* in the development and function of mesodermal tissues and brain. We provide evidence that *Igfbp5* encoding a component of IGF signaling is a direct Six5-target. Moreover, the overall expression level of *Igfbp5* was decreased in *Six5*-deficient mouse fibroblasts, and the response of human *IGFBP5* to MyoD-induced muscle conversion was altered in cells of DM1 patients. Our results not only identify Six5 as an activator that directs *Igfbp5* expression but also suggest that reduced *SIX5* expression in DM1 might contribute to specific aspects of the DM1 phenotype.

INTRODUCTION

Myotonic dystrophy 1 (DM1, MIM160900) is the most common inherited neuromuscular disease in adults, characterized by myotonia, muscle wasting and weakness, cataract, insulin resistance, cardiac conduction defect, testicular atrophy, and mental impairment (1). DM1 is caused by the expansion of an unstable CTG repeat located in the 3′ untranslated region (UTR) of DMPK on 19q13.3 (2–4). The consequence of the repeat expansion includes abnormal splicing and transport of DMPK transcripts, which result in a decrease in DMPK protein levels (5) and sequestration or induction of RNA-binding proteins by transcribed, expanded CUG repeats (6). In addition, the repeat lies immediately 5′ to the regulatory region of the homeobox gene SIX5 (7–9), and repeat expansion causes the loss of a DNase I-hypersensitive site in the region and suppression of SIX5 expression (8,10,11).

SIX5 belongs to the Six class of homeobox genes (12). The gene is expressed in most of the tissues affected by DM1, such as skeletal and smooth muscles, central and peripheral nervous systems, and adult lens (7,9,13–18). As such, haploinsufficiency of SIX5 is thought to contribute to the development of some of the symptoms. Indeed, two independent lines of Six5-deficient mice have been produced and shown to develop cataract, suggesting that reduced SIX5 function underlies cataract phenotype in DM1 (15,17). While other phenotypes associated with DM1 are not apparent in those animal models, it is possible that SIX5 deficiency, in conjunction with other molecular defects resulting from the repeat expansion, is involved in the pathogenesis of DM1. For example, the fact that Six5 can activate transcription of the myogenin (Myog) gene encoding a myogenic basic helix–loop–helix (bHLH) protein through the MEF3 site, an essential promoter element required for the lineage-specific expression (19,20), may implicate SIX5 dysfunction in muscle phenotypes such as the fusion delay and fiber maturation defects seen in congenital DM1 cases (21,22). This notion is corroborated by the observation that mutations of D-Six4, the Drosophila counterpart of SIX5, cause a myoblast fusion defect and testicular/ovarian reduction (23). However, the phenotype of transgenic mice expressing an expanded CTG

*To whom correspondence should be addressed. Tel: + 81 285 58 7311; Fax: + 81 285 44 5476; Email: kkawakam@jichi.ac.jp

repeat (24) and the molecular characterization of DM2, which shows almost identical phenotype with DM1 (25), cast some doubt on the extent of involvement of SIX5 in the pathogenesis of DM1. This is partly due to insufficient characterization of SIX5, particularly of downstream transcriptional targets regulated by the SIX5 homeodomain protein. Identification of transcriptional targets for SIX5 is therefore essential to understand the involvement of SIX5 deficiency in DM1.

Known downstream target genes for SIX5 include the aforementioned Myog identified by inference and Atp1a1 encoding the α1 subunit of Na, K-ATPase, which was identified biochemically as a target gene for Six family proteins (13,26,27). A recent whole-genome PCR-based screening identified the 5′ flanking sequence of DRD5 encoding the dopamine 5D receptor and several genomic DNA fragments as potential target sequences (28). The other way to screen SIX5-target genes is to overexpress SIX5 and detect genes whose expression levels change (increase or decrease) by overexpression. SIX5 is a transcription factor characterized by the presence of the Six domain (SD) and Six-type homeodomain (HD), both of which are required for specific DNA binding and interaction with Eya transcription coactivators (13,19). The SD is also known to interact with the Groucho family of corepressors (29), suggesting that SIX5 can act both as an activator and as a repressor.

In the present study, we sought to identify SIX5-target genes. A constitutively active form of mouse Six5 (VP16–Six5wt) was overexpressed in P19 embryonal carcinoma cells by adenovirus-mediated gene transfer and a group of genes induced by the treatment was detected by hybridization to cDNA arrays. To minimize background resulting from adenovirus infection and/or overexpression of the full-length Six5 and VP16 activation domain, both of which interact with various nuclear and cytoplasmic proteins, a HD mutant protein (VP16–Six5W241R) was overexpressed in the control set. Among 21 potential target genes, Igfbp5 and Igf2, which are involved in insulin-like growth factor (IGF) signaling, were subjected to further analyses in order to validate our approach. A Six5-binding site in the Igfbp5 promoter was determined by reporter gene assays and in vitro binding experiments. Expression levels of Igfbp5 and Igf2 were reduced in $Six5^{-/-}$ fibroblasts and expression of human IGFBP5 was altered in cells of DM1 patients. These results not only identify Six5 as one of the activators directly involved in Igfbp5 expression but also suggest that reduced SIX5 expression in DM1 might contribute to specific aspects of the DM1 phenotype.

RESULTS

Production of recombinant adenovirus expressing a constitutively active Six5

To construct a constitutively active form of Six5, a strong transcription activation domain of herpes simplex virus VP16 was fused to full-length wild-type Six5 (Fig. 1A). As a control, a point mutation (W241R) was introduced in the HD. The site is conserved in the Six family as well as in many other HD proteins, and is thought to play a critical role in the structure and specific DNA binding of the proteins (30). When plasmids expressing VP16–Six5wt and VP16–Six5W241R were transiently transfected into COS7 cells, recombinant proteins were detected by anti-Six5 and anti-VP16 antibodies (Fig. 1B). The W241R mutation did not affect nuclear localization (data not shown). In gel retardation assays, nuclear extracts from COS7 cells expressing VP16–Six5wt contained proteins that bind to an oligonucleotide probe corresponding to the C3 element in the proximal promoter of rat Atp1a1(27) (Fig. 1C, lane 3). The formation of the protein–DNA complex was interfered with by adding an excess amount of cold C3 oligonucleotide (lane 4) but not by a mutagenized oligonucleotide (lane 5). The protein–DNA complex was bound to anti-Six5 (lane 6) and anti-VP16 (data not shown) antibodies, resulting in the formation of a supershifted complex. Thus, VP16–Six5wt specifically binds to the C3 element. In contrast, VP16–Six5W241R retains only a residual activity to bind to the probe (lane 2).

We then constructed recombinant adenoviruses encoding VP16–Six5wt and VP16–Six5W241R in order to overexpress these two proteins. As shown in Figure 1D, recombinant proteins were readily detectable even in whole-cell extracts prepared from cells infected with two recombinant adenoviruses, AxCAwt VP16–Six5wt and AxCAwt VP16–Six5W241R.

To assess the effectiveness of our experimental design in detecting genes regulated by Six5, we analyzed Myog expression in C2C12 mouse myoblasts. As shown in Figure 1E, Myog expression levels were higher in myoblasts infected with AxCAwt VP16–Six5wt than in those infected with AxCAwt VP16–Six5W241R both at 14 and 38 h. In addition, VP16–Six5wt overexpression specifically activated luciferase reporters driven by the Myog promoter (pGL3MG-185) and a synthetic promoter (pTKW4FLF) (19) (data not shown).

Identification of potential downstream target genes for Six5

In our initial attempt to identify potential Six5-target genes, we chose P19 embryonal carcinoma cells to overexpress VP16–Six5wt because they express endogenous Six5 (7). Another reason for selecting these cells is their multipotent property to differentiate into various cell types, including skeletal and cardiac muscles and neurons (31,32).

A medium-scale expression profiling was performed using $poly(A)^+$ RNA samples prepared from P19 cells that were infected with AxCAwt VP16–Six5wt and AxCAwt VP16–Six5W241R and cultured for 24 h. Hybridization of a pair of mouse cDNA macroarrays with probes synthesized using the two RNA samples allowed us to identify 21 genes (1.8%) out of 1176 genes spotted on the array as potential Six5-target genes whose expression levels were more than 2-fold higher in AxCAwt VP16–Six5wt-infected P19 cells than in AxCAwt VP16–Six5W241R-infected cells (Table 1). The 21 potential target genes could be classified into the following categories according to the encoded proteins: transcription factors (Ebf2, Six2, Dermo1, Mesp2, Sim1, Six4, Mdfi, Foxd4 and Pura), signaling molecules and receptors (Igfbp5, Wnt4, Igf2, Sarp1, Fzd8 and Ptn), neurotransmitter receptor and transporter (Gabt3 and Htr3a) and those with other functions (Col9a2, Krt1-18, Pltp and St14). It is notable that four of nine transcription factors identified are bHLH proteins (Ebf2,

Dermo1, Mesp2 and Sim1) and Mdfi is known to interact with bHLH proteins. Remarkably, 10 genes are expressed in mesodermal tissues, including developing somites and skeletal muscle (Six2, Dermo1, Mesp2, Sim1, Six4, Mdfi, Igfbp5, Igf2, Sarp1 and Fzd8) (33–42), and seven genes are expressed in neural tissues, including the neural tube, brain and meninges (Ebf2, Sim1, Foxd4, Wnt4, Fzd8, Gabt3 and Htr3a) (36,43–47). Among the 21 potential targets, the expression levels of Igfbp5,

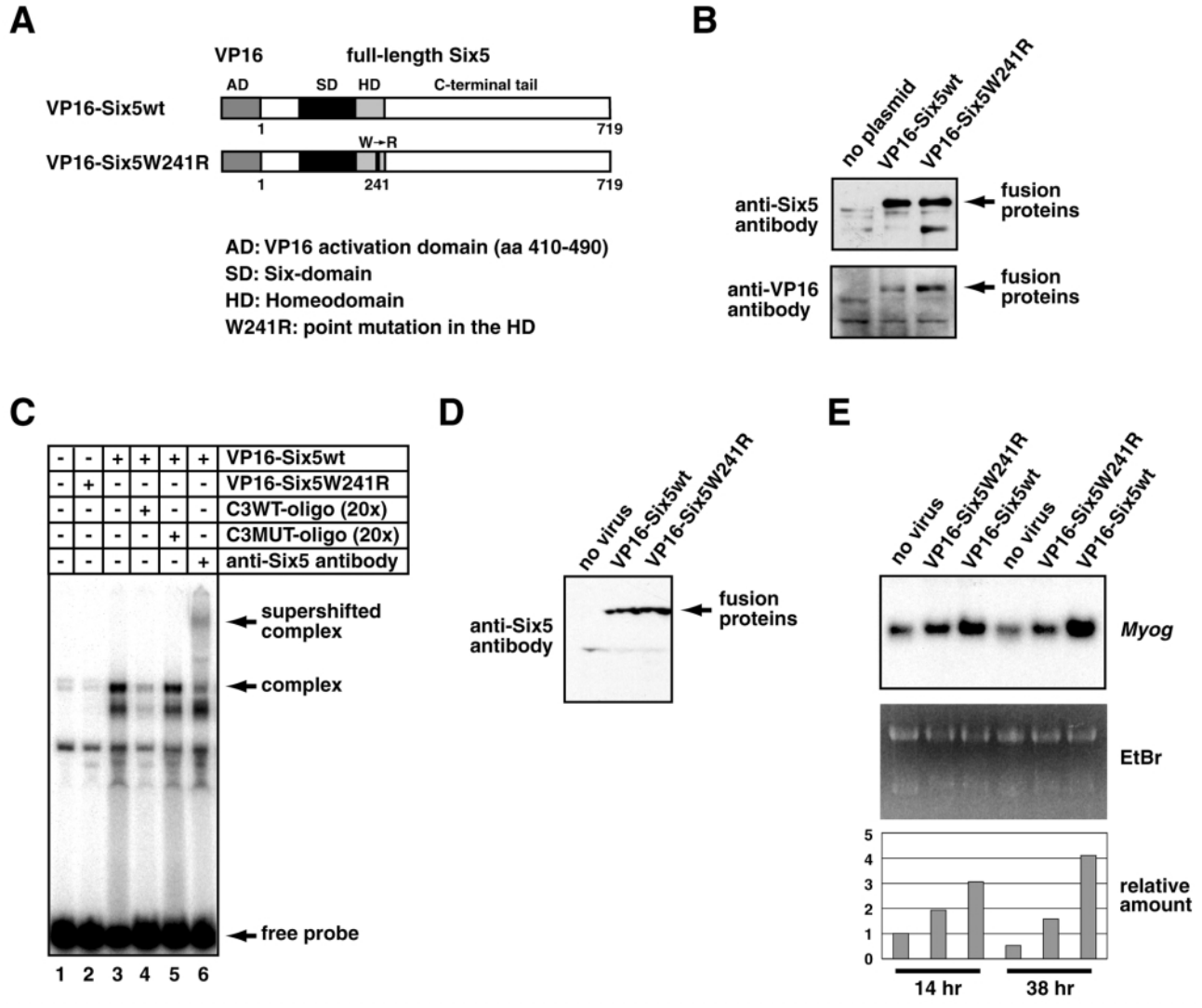

Figure 1. Production of recombinant adenovirus expressing VP16–Six5wt. (A) Structures of VP16–Six5wt and VP16–Six5W241R. Six5 (719 amino acids) is characterized by the Six domain (SD), the Six-type homeodomain (HD) and a long C-terminal tail. A strong activation domain of VP16 (VP16 AD, 81 amino acids) was fused to full-length, wild-type Six5 (Six5wt) and a mutagenized Six5 (Six5W241R). The W241R changes conserved Trp241 in the HD to Arg. (B) Expression of VP16–Six5wt in transfected cells. COS7 cells were transfected with plasmids expressing VP16–Six5wt and VP16–Six5W241R and nuclear extracts were prepared after 48 h. Recombinant proteins were detected by western blot using anti-Six5 (top) and anti-VP16 (bottom) antibodies. Nuclear extracts from non-transfected COS7 cells were used as a control (no plasmid). (C) VP16–Six5W241R is defective in DNA binding. Nuclear extracts prepared from COS7 cells transfected with pCS2+ VP16–Six5wt (lanes 3–6) and pCS2+ VP16–Six5W241R (lane 2) and non-transfected control cells (lane 1) were used to assess the capacity of expressed recombinant proteins to bind the C3WT oligonucleotide (C3WT-oligo) probe by gel retardation assays. To demonstrate a specific binding of expressed VP16–Six5wt to the labeled probe, 20-fold excess amounts of cold C3WT-oligo (lane 4), a mutagenized oligonucleotide (C3MUT-oligo, lane 5) and anti-Six5 antibody (lane 6) were added to the reaction mixtures. C3WT-oligo (lane 4) but not C3MUT-oligo (lane 5) interfered with the formation of the protein–DNA complex. The protein–DNA complex was bound to anti-Six5 antibody, resulting in the formation of a supershifted complex (lane 6). Positions of bands corresponding to retarded protein–DNA complexes and free probe are indicated. (D) Expression of VP16–Six5wt in adenovirus-infected cells. COS7 cells were infected with recombinant adenoviruses expressing VP16–Six5wt and VP16–Six5W241R [at a multiplicity infection (m.o.i.) of 100 plque-forming units (PFU)/cell], and whole-cell extracts were prepared after 24 h. Recombinant proteins were detected by western blot using anti-Six5 antibody. Nuclear extracts from non-infected cells were used as control (no virus). (E) Activation of Myog expression by VP16–Six5wt. C2C12 myoblasts were infected with adenoviruses expressing VP16–Six5wt and VP16–Six5W241R (at an m.o.i of 200 PFU/cell), and total RNAs were isolated at 14 and 38 h after infection. The expression of Myog was detected by northern blot using a ^{32}P-labeled probe (top). An ethidium bromide (EtBr)-stained gel showing rRNA bands demonstrates integrity and equal loading (10 µg/lane) of RNA (middle). Relative amounts of Myog transcripts, measured from the above autoradiogram, are shown (bottom). RNA prepared from non-infected cells was used as control (no virus).

Table 1. List of genes upregulated by VP16–Six5wt overexpression in P19 cells

	Symbol	Potential Six5-binding site		Accession no.
		TCARRTTNC	TCARRNNNY	
Transcription factor:				
dermis expressed 1	Dermo1			(–)
early B-cell factor 2	Ebf2			(–)
forkhead box D4	Foxd4	No	− 294*, 138	X86368
mesoderm posterior 2	Mesp2			(–)
MyoD family inhibitor	Mdfi			(–)
purine rich element-binding protein A	Pura			(–)
single-minded 1	Sim1	− 1534	− 3599, − 2619, − 2205 − 1870	AB013484
sine oculis-related homeobox 2 homolog	Six2	No	− 355, 823, 1383* 1445, 1635*, 1868*	AF136939 (h)
sine oculis-related homeobox 4 homolog	Six4	− 100*	− 941*, − 731, − 567* − 432	AB024688
Signaling molecule/receptor:				
frizzled homolog 8	Fzd8			(–)
insulin-like growth factor 2	Igf2	− 33*(P1)	− 223(P1), − 54*(P1) 91(P3)	X71918 X71920
insulin-like growth factor-binding protein 5	Igfbp5	− 70	− 2605, − 2414*, − 1871 − 1779*, − 1154	U02023
pleiotrophin	Ptn	− 1413*	− 1350*, − 1200*	X65451 (h)
secreted apoptosis-related protein 1	Sarp1			(–)
wingless-related MMTV integration site 4	Wnt4	− 2682*	− 3794, − 3574*, − 2996 − 2955*, − 1400, − 849 − 674*	AF414100
Neurotransmitter receptor/transporter:				
gamma-aminobutyric acid (GABA-A) transporter 3	Gabt3			(–)
5-hydroxytryptamine (serotonin) receptor 3A	Htr3a			(–)
Others:				
keratin complex 1, acidic, gene 18	Krt1-18	No	− 896, − 345	Y00217
phospholipid transfer protein	Pltp	No	No	U58941
procollagen, type IX, alpha 2	Col9a2	No	− 2336*, − 402*, − 107* − 32*	Z22923
suppression of tumorigenicity 14	St14			(–)

Listed are genes with expression levels > 2-fold higher in AxCAwt VP16–Six5wt-infected P19 cells than in AxCAwt VP16–Six5W241R-infected cells in two independent experiments. The gene name, gene symbol and functional categories are shown. The positions of potential Six5-binding sites present in promoter regions, sequences flanking 5′ ends of longest cDNA sequences, 5′ untranslated region and introns of potential target genes (given with GenBank accession numbers) are also shown. The position numbering is relative to transcription start sites or 5′ ends of cDNA sequences. Promoter sequences were surveyed first for the consensus sequence TCARRTTNC (1 in 16384 bp) and then for a less strict consensus TCARRNNNY (1 in 512 bp). (h) indicates that the mouse promoter sequence was unavailable and the orthologous human gene was used and surveyed for potential Six5-binding sites. (–) indicates that both mouse and human promoter sequences were unavailable. Asterisks indicate the potential Six5-binding sites found in the reverse orientation relative to the transcription start site. While Igf2 is transcribed from three different promoters (P1–P3) (59), potential Six5-binding sites were found in P1 and P3.

Igf2, Six2 and Mdfi were examined by northern blot analysis to confirm the array result (Fig. 2 and data not shown). Igfbp5 expression was almost undetectable in uninfected control and AxCAwt VP16–Six5W241R-infected cells but markedly induced (>10-fold) in AxCAwt VP16–Six5wt-infected cells. In contrast, although it was clear that Igf2 expression was induced after AxCAwt VP16–Six5wt infection, the induction ratio was not very high (approximately 3-fold). The induction ratios for Six2 and Mdfi were 17.4- and 1.9-fold, respectively (data not shown).

Direct activation of the Igfbp5 promoter by Six5

We focused on Igfbp5 and Igf2 as two representative potential target genes regulated by Six5. Igfbp5 encodes IGF-binding protein 5 (IGFBP-5), which can either inhibit or potentiate IGF signaling by binding to IGF-I and IGF-II (48). Among the major sites of Igfbp5 expression during embryogenesis are somites and muscle precursor cells (39). Igf2 encoding IGF-II is abundantly expressed in the early embryonic mesoderm and its derivatives such as the developing somites and heart (40,49), whereas its expression in the adult is mostly confined to the choroid plexus and leptomeninges (50).

Regulation of Igfbp5 expression has been analyzed in some detail (51–55). Such studies showed that the proximal promoter is essential for the basal transcription and is also required for response to various signaling molecules. To determine whether the Igfbp5 promoter is directly regulated by Six5, we tested the response of luciferase reporter constructs driven by 5′ flanking fragments of Igfbp5 to VP16–Six5wt and VP16–Six5W241R, which is defective in DNA binding (Fig. 1C). As shown in Figure 3A, VP16–Six5wt had a very strong inductive effect on

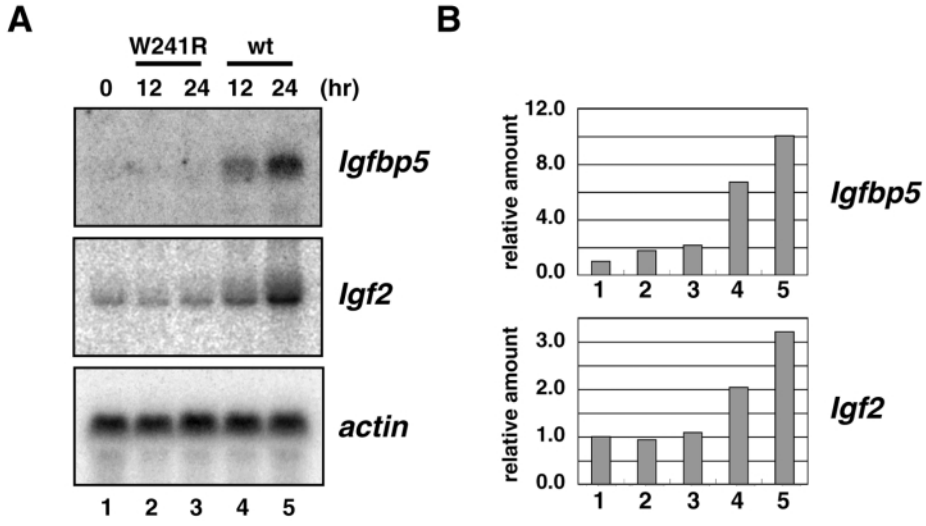

Figure 2. Activation of Igfbp5 and Igf2 expression by VP16–Six5wt. (A) Northern blot analysis. P19 cells were infected with adenoviruses expressing VP16–Six5wt (lanes 4, 5) and VP16–Six5W241R (lanes 2, 3) at an m.o.i. of 200 PFU/cell, and total RNAs were isolated at 12 and 24 h after infection. The expression levels of Igfbp5, Igf2 and control β-actin were analyzed. RNA prepared from non-infected cells was used as a control (lane 1). (B) Relative amounts of Igfbp5 and Igf2 transcripts. The hybridization signals of Igfbp5 and Igf2 transcripts were quantitated after normalization to β-actin signals. Column numbering corresponds to the lane numbering used in (A).

the longest construct, Igfbp5–2.9k Luc, exhibiting more than a 300-fold higher luciferase activity compared with the promoterless construct pGL3 in COS7 cells. Similarly, VP16–Six5wt activated luciferase expression from the following deletion constructs: Igfbp5–1.4k Luc, Igfbp5–160 Luc and Igfbp5–105 Luc. We noted, however, that larger deletions resulted in rather progressive decrease in Six5 responsiveness (expressed as the Six5wt : Six5W241R ratio): Igfbp5–2.9k Luc, 52.9; Igfbp5–1.4k Luc, 46.4; Igfbp5–160 Luc, 44.0; Igfbp5–105 Luc, 35.8. This suggests the possible involvement of multiple regulatory elements. Deletion of a region between positions −105 and −69 virtually abolished Six5-responsiveness from luciferase reporters (Fig. 3A; compare Igfbp5–105 Luc and Igfbp5–69 Luc), suggesting that the 37 bp deletion either removed or disrupted a sequence(s) critical for Six5-induced activation. Results obtained using P19 cells also showed a similar effect of the 37 bp deletion (data not shown).

Characterization of a Six5-binding site in the Igfbp5 promoter

To explore the possibility that a Six5-binding site(s) lies within 105 bp of the 5′ flanking region, we next performed gel retardation assays using a BseDI restriction fragment (positions −105 to −39, Fig. 3B, probe 1) and shorter oligonucleotide probes (Fig. 3B, probes 2–4). GST–Six5 bound to probe 1 as well as probe 3, which corresponds to positions −95 to −58 (Fig. 3C). Binding to probes 2 and 4 was not detectable. The results suggest that Six5 activates Igfbp5 expression directly through Six5-binding elements and that one of the elements is located in the region between positions −95 and −58.

We next sought to identify the Six5-binding sequence in the proximal promoter employing in vitro binding experiments (Fig. 4). DNase I footprinting and methylation interference assays revealed that GST–Six5 could bind to the sequence GCTCAAATTGC located between −72 and −62 (Fig. 4A and B). The Six5-binding sequence was perfectly conserved in the corresponding rat and human genes (Fig. 4C). In addition, comparison with two previously characterized Six5-target sites, the C3 element in the Atp1a1 promoter and the MEF3 site in the Myog promoter (19,20), and the MEF3 consensus KSSTCAGGNNNY (56) allowed us to extract a consensus Six5-binding sequence, TCARRTTNC (Fig. 4C).

Expression of Six5-target genes in Six5 $^{-/-}$ fibroblasts

To confirm that Six5 is involved in the regulation of downstream target genes identified in our screening, we examined the expression levels of Igfbp5 and Igf2 in fibroblasts prepared from homozygous Six5-deficient embryos (15). As shown in Fig. 5, overall expression levels of Igfbp5 and Igf2 in Six5 $^{-/-}$ fibroblasts were decreased compared with wild-type fibroblasts. Interestingly, the responses to various stimuli such as serum-starvation (Fig. 5, lanes 2 and 3), IGF-II (Fig. 5, lane 4) and IGF-I (data not shown) were essentially similar between the two types of fibroblasts. Both IGF-I and IGF-II have been shown to increase Igfbp5 mRNA level (57,58). These data suggest a positive regulatory role of Six5 in the basal transcription of Igfbp5 and Igf2, rather than the activated transcription in response to IGFs in cultured embryonic fibroblasts.

Cooperative activation of Igfbp5 expression by Six5 and Eya3

Although we have shown that a constitutively active Six5 (VP16–Six5wt) can activate reporter gene expression driven by the Igfbp5 promoter and that Igfbp5 expression level is decreased in cultured Six5 $^{-/-}$ fibroblasts, the mechanism by which Six5 regulates Igfbp5 transcription is unclear. In this

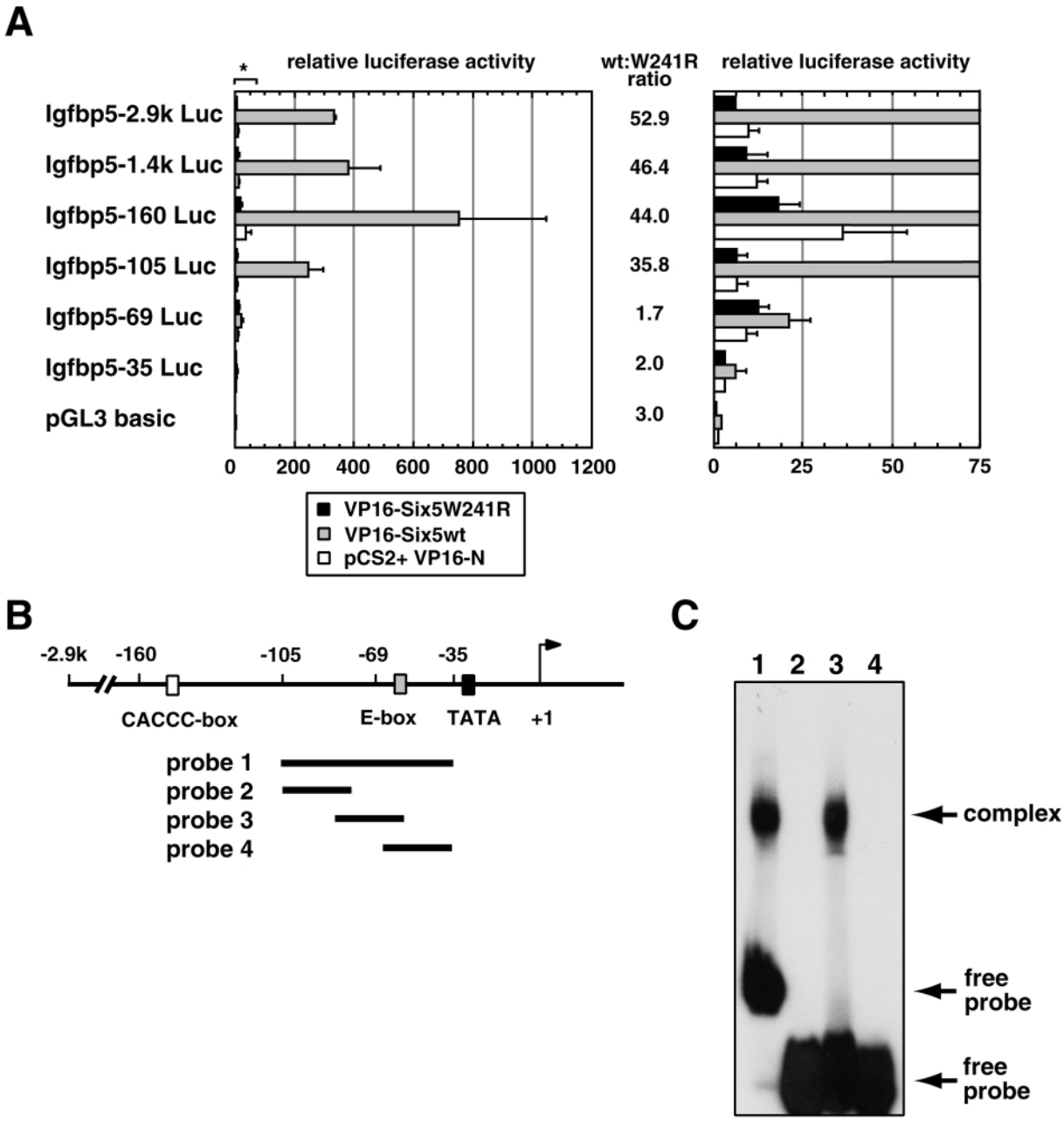

Figure 3. Activation of the Igfbp5 promoter by VP16–Six5wt. (A) Effect of VP16–Six5wt on the Igfbp5 promoter. Response of various luciferase reporter constructs driven by the Igfbp5 promoter fragments to co-transfection of pCS2+ VP16-N, VP16–Six5wt and VP16–Six5W241R was analyzed using COS7 cells. Luciferase activity values are normalized to β-galactosidase activity and expressed relative to the value obtained by a combination of the empty pCS2+ VP16-N and pGL3 basic plasmids. Inductive effects of VP16–Six5wt (wt) on reporter constructs relative to VP16–Six5W241R (W241R) are shown on the right (wt : W241R ratio). Data are mean ± SD fold activation from three independent experiments (each performed in duplicate). The part indicated by the asterisk in the left panel is horizontally magnified to show clearly columns representing low activity (right panel). (B) Structure of the Igfbp5 promoter. Schematic representation of the proximal Igfbp5 promoter with the positions of previously characterized cis-regulatory elements (CACCC-box, E-box and TATA-box) and a major transcription start site (+1). Solid bars at the bottom show positions of a BseDI fragment (probe 1) and oligonucleotide (probes 2–4) used as probes for gel retardation assay in (C). (C) Binding of Six5 to the Igfbp5 promoter. A gel retardation assay was performed using bacterially expressed GST–Six5 and the probes indicated in (B): lane 1, probe 1; lane 2, probe 2; lane 3, probe 3; lane 4, probe 4.

regard, cooperative activation by Six5 and Eya3 has been demonstrated in Myog transcription (19). We examined whether Six5 acts cooperatively with Eya3 to activate transcription of Igfbp5. Transient transfection of the plasmid expressing FLAG–Six5 showed an activating effect on Igfbp5–160 Luc, one of the Six5-responsive reporter constructs (Fig. 3A) in COS7 cells (Fig. 6, column 2). Coexpression of HA-tagged Eya3 resulted in more than 4-fold induction relative to FLAG–Six5

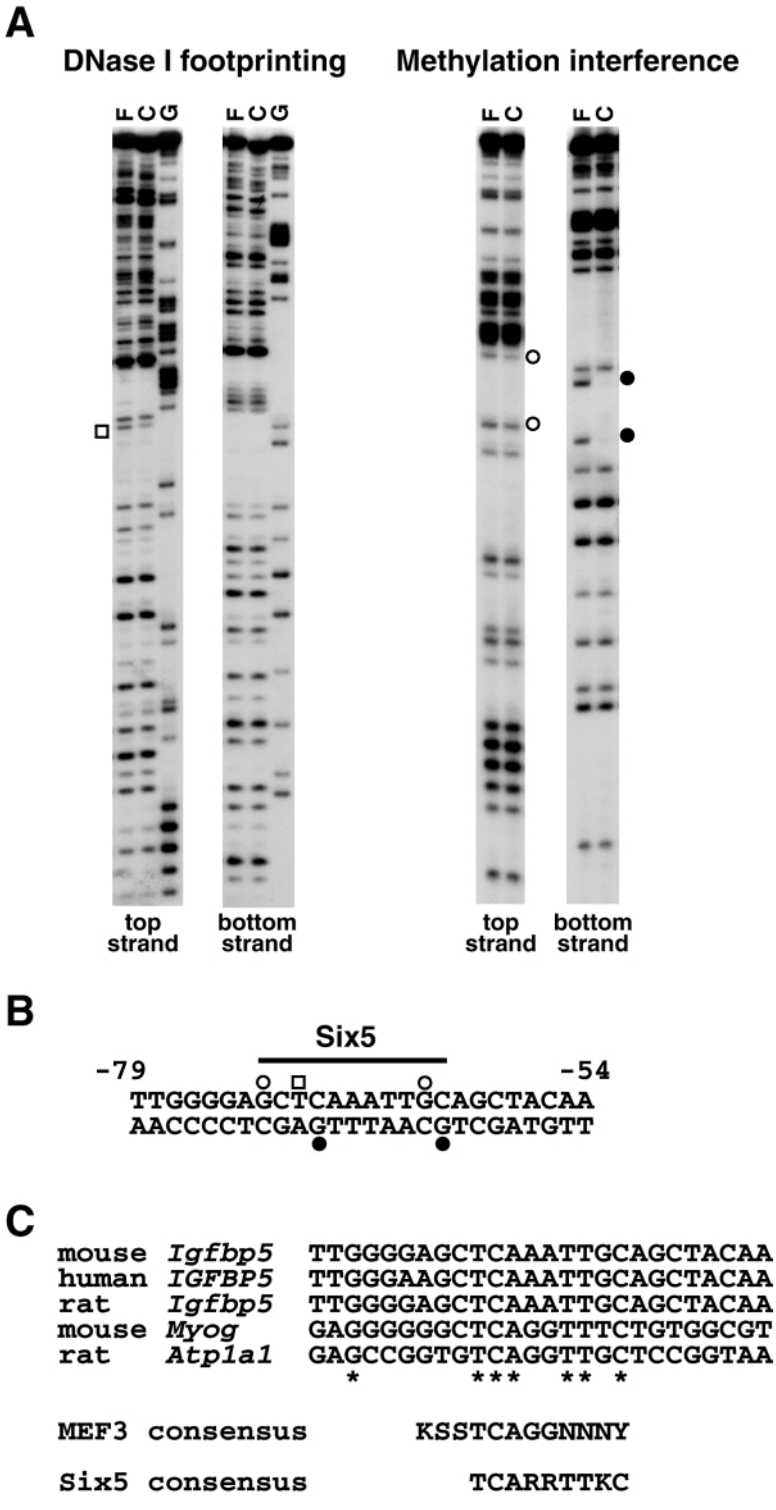

Figure 4. Identification of the Six5-binding site in the Igfbp5 promoter. (A) The result of DNase I footprinting and methylation interference assays. GST–Six5 and ^{32}P-labeled Igfbp5 promoter fragments were used. The top and bottom strands indicate the strands that were end-labeled with ^{32}P. Open square, a T residue protected weakly in DNase I footprinting; closed circles, methylation of these G residues strongly interfered Six5 binding; open circles, G residues that weakly interfered with Six5 binding; lane C, DNA-probes in the Six5-bound complexes; lane F, free DNA probes separated from the Six5–DNA complexes; lane G, G ladders of Maxam–Gilbert sequencing. (B) Summary of the DNase I footprinting and methylation interference assays shown in (A). The solid bar at the top indicates a predicted Six5-binding site. (C) A consensus Six5-binding site. Alignment of Six5-binding sites from the mouse Igfbp5 promoter (this study), corresponding sequences from human IGFBP5 (GenBank accession no. U20271) and rat Igfbp5 (L08275) promoters, the C3 element of rat Atp1a1 promoter (X53233) and the MEF3 site of the mouse Myog promoter (M95800). Asterisks indicate identical residues in the aligned sequences. The MEF3 consensus sequence KSSTCAGGNNNY (56) and a proposed consensus Six5-binding sequence, TCARRTTNC, are shown below.

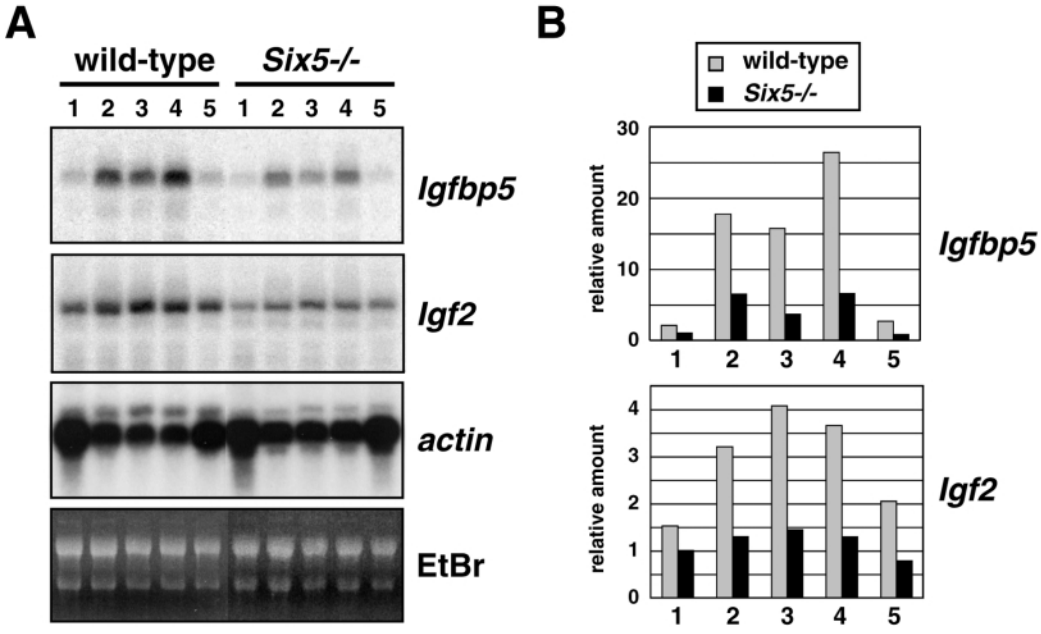

Figure 5. Igfbp5 and Igf2 expression levels in Six5-deficient fibroblasts. (A) Northern blot analysis. Fibroblasts prepared from wild-type (wild-type) and Six5-deficient (Six5 $^{-/-}$) mouse embryos were cultured to confluence (lane 1), serum-starved for 24 h (lane 2) and then treated with several different culture conditions for 12 h (lane 3, serum-free DMEM; lane 4, 100 ng/ml IGF-II, lane 5, 10% FCS). The expression levels of Igfbp5, Igf2 and control β-actin (bottom) were analyzed. The EtBr-stained gel showing rRNA bands demonstrates the integrity of loaded RNA (6 μg/lane). (B) Relative amounts of Igfbp5 and Igf2 transcripts. The hybridization signals of Igfbp5 and Igf2 transcripts were quantitated after normalization to the intensity of 28S rRNA bands because β-actin hybridization signals showed significant differences among samples. Column numbering corresponds to the lane numbering used in (A).

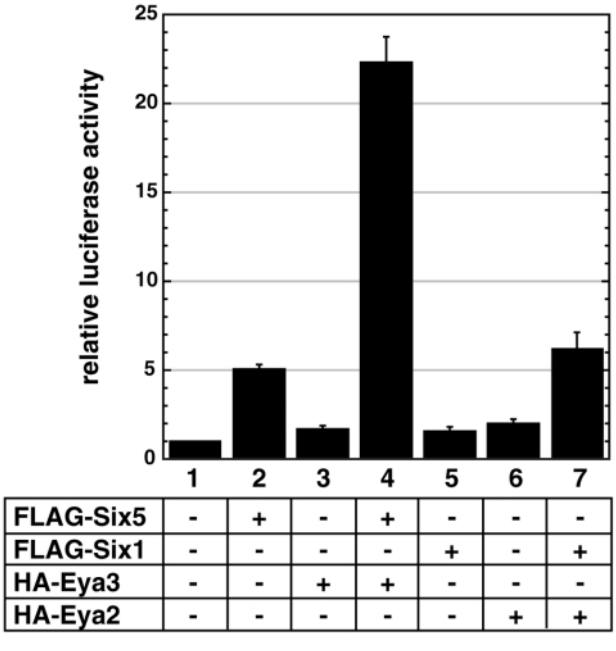

Figure 6. Cooperative activation of the Igfbp5 promoter by Six5 and Eya3. Effects of FLAG-tagged Six5 (FLAG–Six5, columns 2 and 4), Six1 (FLAG–Six1, columns 5 and 7) in combination with HA-tagged Eya3 (HA–Eya3, columns 3 and 4) and Eya2 (HA–Eya2, columns 6 and 7) on the luciferase reporter Igfbp5–160 Luc were analyzed using COS7 cells. Luciferase activity values are normalized to β-galactosidase activity and expressed relative to the value obtained from control non-transfected cells (column 1). Data are mean ± SD fold activation from three independent experiments (each performed in duplicate).

alone (columns 2 and 4). Taken together, these results suggest that Six5 is one of the activators that bind to the proximal promoter of Igfbp5 and that the activation mechanism by Six5 may involve interaction with the Eya3 coactivator. We also found that Six1, a member of the Six family proteins expressed in tissues of mesodermal origin, including the developing somites (33), is capable of activating the Igfbp5 promoter when expressed with Eya2 (Fig. 6, columns 5 and 7). This suggests that other Six family members in conjunction with coexpressed Eya proteins may also play a role in Igfbp5 expression in certain circumstances.

Expression of IGFBP5 in DM1 fibroblasts and skeletal muscles

In DM1 patients, a decrease in SIX5 expression caused by the CTG repeat expansion is likely to result in reduced expression of SIX5-target genes. If such is the case, genes identified in our screening, including IGFBP5, should exhibit low expression levels in cells of DM1 patients. However, considering that the repeat expansion also disturbs DMPK expression, RNA metabolism and intracellular signaling machinery, the effect on a given target gene may not be so simple. To examine whether IGFBP5 expression is affected by the repeat expansion, we isolated fibroblasts from three independent DM1 patients, each with an expansion of at least 1000 repeats, and examined IGFBP5 expression before and after MyoD-induced skeletal muscle conversion by northern blot analysis (Fig. 7). In two different DM1 fibroblast, the expression levels of IGFBP5 increased following skeletal muscle conversion but the

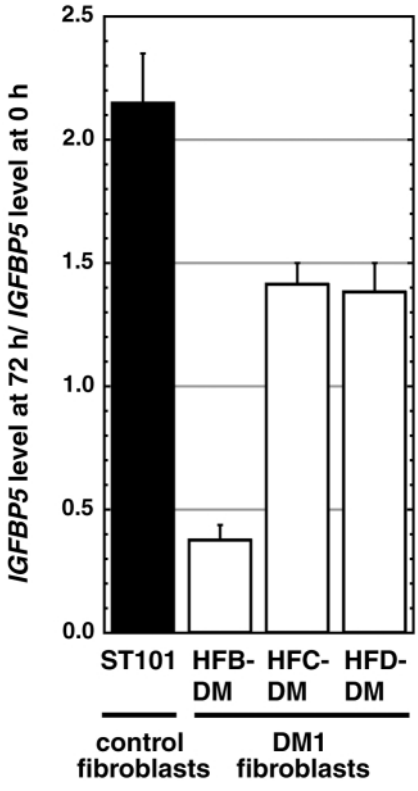

Figure 7. IGFBP5 expression levels in DM1 cells. Fibroblasts prepared from adults with or without DM1 were infected with retroviral vectors pBABE MyoD-ER (ST101, HFB-DM and HFD-DM) or pLXSN MyoD-ER (HFC-DM). Infected cells were split in two, cultured to confluence and processed for RNA extraction before (0 h) or after treatment with a differentiation medium supplemented with 10^{-7} M β-estradiol for 72 h. Expression of IGFBP5 was detected by northern blot and quantitated. Changes in IGFBP5 expression levels following skeletal muscle conversion (IGFBP5 level at 72 h/IGFBP5 level at 0 h) were calculated for each cell line. Mean values from two independent experiments are shown, with the bars indicating the higher values.

differentiation-dependent rises in IGFBP5 levels (HFC-DM, 1.41-fold; HFD-DM, 1.38-fold) were lower compared to that of wild-type fibroblasts (ST101, 2.15-fold). In another DM1 fibroblast, the expression level of IGFBP5 decreased during skeletal muscle conversion (HFB-DM, 0.38-fold). These results seem to indicate that the response of IGFBP5 to muscle conversion is altered in cells of DM1 patients.

DISCUSSION

We have described here an experimental system to identify downstream transcriptional targets of Six5, the mouse orthologue of SIX5 implicated in the pathogenesis of DM1. Using overexpression of a constitutively active Six5 (VP16-Six5wt) and expression profiling by hybridization of cDNA expression arrays, 21 genes whose expression level increased after VP16–Six5wt overexpression were identified as potential target genes. Genes expressed in the developing somites, skeletal muscle, developing brain and meninges comprise the majority, consistent with Six5 expression pattern. The array result on Igfbp5 was confirmed by northern blot analysis, reporter gene assays, in vitro binding experiments and expression analysis using Six5 $^{-/-}$ cells. Finally, we showed that the expression of IGFBP5 was altered in cells of DM1 patients.

Identification of downstream target genes and binding elements for Six5

Recently, a whole genome PCR-based screening was performed to identify SIX5-target genes (28). The method, which utilized the DNA-binding domain of SIX5 fused to GST to survey genomic DNA fragments with potential Six5-binding sites in vitro, picked up several DNA fragments, leading to the identification of DRD5 as a potential target. In this study, we set up a different model system to screen potential target genes in a cellular context. We focused on genes induced by VP16–Six5wt overexpression because recombinant Six5 proteins fused to repression domains from Drosophila Engrailed and human AREB6 did not act as a repressor on various Six5-responsive reporters (data not shown). As to 21 potential Six5-target genes, we cannot exclude the possibility that VP16–Six5wt has the potential to regulate immediate early genes and thus overexpression of Six5 could lead to rapid changes in expression of genes that are not directly regulated by Six5. However, a consensus binding sequence for Six5, TCARRTTNC, predicted using a binding site in the Igfbp5 promoter, was found in five of 10 other potential target genes, including Igf2, whose 5′ flanking and non-coding sequences are deposited in the data bank (Table 1), and in two of the SIX5-binding genomic DNA fragments, AF242570 and AF242573, isolated in the aforementioned study (28). When a less strict consensus, TCARRNNNY, was applied, the sequence was found in SIX5-binding DNA fragments at high-frequency except AF242572, suggesting that both methods could effectively detect Six5/SIX5 target genes.

In Table 2, we list positions of potential Six5-binding sites found in known Six5 targets (Atp1a1 and Myog) and genes implicated in DM pathogenesis [Insr, Tnnt2, Clcn1 and Cnbp (the mouse orthologue of ZNF9)]. Among these, Myog and Insr encoding the insulin receptor were present in the cDNA expression array used in our study. Induction ratios of Insr and Myog by VP16–Six5wt overexpression in P19 cells were 1.1 and 0.88, respectively. Drd5 was not present on the array. It remains to be established whether any of the potential sites listed in Table 2 (except the C3 element in Atp1a1 and the MEF3 site in Myog) are involved in Six5-mediated transcriptional regulation.

Igfbp5 as a direct target gene for Six5

We focused on Igfbp5 and Igf2 as two representative potential target genes. Changes in the response of IGFBP5 to MyoD-induced muscle conversion in three independent DM1 fibroblasts may partly result from reduced SIX5 expression. As to Igf2, we did not test how Six5 activates the gene, since its expression is regulated not only by three different promoters (59) but also by multiple downstream enhancers that are shared with the closely linked H19 gene (60), making it difficult to assess the effect of Six5 on transcription using ordinary reporter gene assays. However, given an increase in Igf2 expression level after 12 h of Six5 overexpression in P19 cells,

Table 2. Six5-binding sites in genes implicated in DM

	Symbol	Potential Six5-binding site		Accession no.
		TCARRTTNC	TCARRNNNY	
Known Six5 target genes:				
ATPase, Na+/K+ transporting, alpha1 polypeptide	Atp1a1	− 736, − 90(C3)	− 1291, − 1236, − 264	X53233 (r)
myogenin	Myog	− 243, − 87(MEF3)	− 1294*	M95800
DM-related genes:				
cellular nucleic acid binding protein (ZNF9 orthologue)	Cnbp	− 2096	− 4165*, − 3255*, − 2285 − 1584, − 1480*, − 1145 − 1078, − 529	AF389886 (h)
chloride channel 1	Clcn1	No	No	Z31375
insulin receptor	Insr	No	− 279*, − 206*, 119 433	M28869
troponin T2, cardiac	Tnnt2	− 3427	− 951*, − 464*	AB052890

Listed are genes known to be regulated by Six5 (known Six5 target genes) and those implicated in DM pathogenesis (DM-related genes). The positions of potential Six5-binding sites present in promoter regions, sequences flanking 5′ ends of longest cDNA sequences and 5′ untranslated region are shown (given with GenBank accession numbers). Known Six5-binding sites (the C3 element in Atp1a1 and the MEF3 site in Myog) are also included. The position numbering is relative to transcription start sites or 5′ ends of cDNA sequences, except for Tnnt2, in which the positions of potential Six5-binding sites are indicated relative to the predicted translation initiation codon. Promoter sequences were surveyed first for the consensus sequence TCARRTTNC and then for a less strict consensus TCARRNNNY. (h) indicates that the mouse promoter sequence was unavailable and the orthologous human gene was used and surveyed for potential Six5-binding sites. (r) indicates that the orthologous rat gene was used and surveyed for potential Six5-binding sites. Asterisks indicate the potential Six5-binding sites found in the reverse orientation relative to the transcription start site.

a decrease in Igf2 expression in Six5 $^{-/-}$ fibroblasts and the presence of potential Six5-binding sites (Table 1), we believe the gene is also a direct target of Six5.

If Six5 directly (or indirectly) activates the expression of Igfbp5 and Igf2, the question arises as to why Six5 $^{-/-}$ mice do not show growth deficiency, a phenotype expected from reduced Igf2 expression (61), or defects in muscle or other mesodermal tissues that express Igf2 and Igfbp5. There are three potential explanations that account for the lack of a clear muscle phenotype in Six5 $^{-/-}$ mice. (i) Other members of the Six family of proteins, such as Six1 and Six4, may compensate for the loss of Six5 in Six5 $^{-/-}$ mice (15,37). We have shown that the Igfbp5 promoter can be activated not only by Six5 but also by Six1. Intriguingly, activation of the skeletal muscle differentiation program by overexpression of PAX3–FKHR results in the induction of Six1, Igf2 and Igfbp5 (62). (ii) Other transcription activators binding to the sequences flanking Six5-binding sites may achieve sufficient expression levels of Igf2 and Igfbp5 required for embryogenesis to proceed normally. It is possible that the effect of Six5 loss on overall expression levels of Igf2 and Igfbp5 became apparent because of the culture conditions used. (iii) A defect in Six5 $^{-/-}$ muscle may only appear during regeneration or after changes in functional loading (overloading and unloading).

Implications for insulin resistance and cataractogenesis

Insulin resistance is a common metabolic abnormality in DM1 patients. The defect was shown to be associated with aberrant regulation of insulin receptor pre-mRNA splicing and proposed to be caused by an increase in CUG-BP (63). While this seems to provide a plausible explanation, it is still possible that a decrease in SIX5 expression also contributes to insulin resistance. It has been reported that recombinant IGF-I can partially circumvent impaired insulin action in cultured DM1 myotubes (64) and improve metabolism and function in the muscle of DM1 patients in a clinical trial (65). Binding of IGFs to both IGF-I and insulin receptors and activation of intracellular targets common to the two receptors are thought to be the molecular basis of the beneficial effects of IGFs (66). As such, changes in the expression level of IGF signaling components such as IGF-II and IGFBP-5 would also modulate insulin action in DM1 patients.

Adult-onset cataract is the most characteristic eye phenotype in DM1. Six5 and SIX5 are expressed in most structures of the eye, including the lens epithelium (13–17), and cataract formation is recapitulated in two independent lines of Six5-deficient mice (15,17). In such mice, homo- and heterozygous losses of Six5 were considered to result in dysregulation of its downstream target genes in the lens and to cause cataracts. While expression of Igf2 and Igfbp5 is detected in the ciliary body, cornea, retina and sclera but not in the lens (67,68), dysregulation of IGF signaling is implicated in cataract formation (69,70). Our study may implicate Six5-target genes encoding proteins such as IGF-II and IGFBP-5 that are secreted from ocular tissues surrounding the lens in cataractogenesis in DM1 patients and in Six5-deficient mice.

The role of Six5 in mesoderm development and brain function

Previous studies have demonstrated a broad expression of Six5 in various tissues. Of the 21 potential Six5-target genes identified, 10 are known to be expressed in the developing somites and skeletal muscles (Six2, Dermo1, Mesp2, Sim1, Six4, Mdfi, Igfbp5, Igf2, Sarp1 and Fzd8). Because it has been established that Six5 directly activates Myog expression through the MEF3 site (19,20), identification of additional genes expressed in muscle-forming mesodermal tissues further implicates a decrease in SIX5 expression in fusion delay and fiber maturation defects in congenital DM1 cases. Our observation that DM1 fibroblasts failed to upregulate IGFBP5

expression to a normal level in response to MyoD-induced muscle conversion can be significant in this regard.

In addition to DRD5 described previously (28), we have found the 5-hydroxytryptamine (serotonin) receptor 3A (Htr3a) and Gabt3 as potential Six5 targets. Thus, Six5 may play roles in normal brain function, and dysregulation of genes encoding these might partly account for the psychological problems in DM1 patients.

Another interesting finding of the present study is that the expression levels of Six2 (> 17-fold) and Six4 (> 3-fold) were elevated by VP16–Six5wt overexpression in P19 cells. Expression analyses of Six genes in Six5- and Six4-deficient mice have previously shown that the loss of Six5 causes a slight increase in the expression of Six1 and Six4 in the skeletal muscle (15), while the loss of Six4 does not affect the expression of Six1, Six2 and Six5 when examined in whole embryos (37). This seems to suggest that Six family genes participate in complex feedback loops employing different members in different cell types.

MATERIALS AND METHODS

Plasmid construction

Plasmids expressing FLAG- and HA-tagged proteins, pfSix5, pHM6 Eya2 and pHM6Eya3, were described previously (19), as was pfSix1 (71). To construct pCS2+ VP16–Six5wt, full-length mouse Six5 cDNA, excised as an XbaI fragment from pfSix5, was cloned into the XbaI site of pCS2+ VP16-N (72). A point mutation, W241R, was introduced in the HD by cassette mutagenesis using the following primers: W241R-FP (mutagenized nucleotides are in lower case, 5′-AGGTCAG-CAACcGGTTtAAaAACCGGCGACA-3′) and its complementary oligonucleotides W241R-RP. The W241R mutation was chosen based on a mutational study of Pit-1 (30).

The mouse Igfbp5 promoter was isolated using the LA PCR in vitro Cloning Kit (TaKaRa). Downstream primers complementary to a published Igfbp5 exon 1 sequence (GenBank accession no. U02023; its transcription start site is numbered +1) were as follows: Igfbp5-RP2 (positions +123 to +147, 5′-TCTTCGAAACCCGCAGGTTCTGCGT-3′) and Igfbp5-RP3 (positions + 97 to + 121, flanked with a SalI restriction site, 5′-TAGTCGACAAGTCCGGAGAAGGGTGCAGACCGA-3′). BglII-digested genomic DNA (BALB/c, Clontech Laboratories) was ligated to a linker supplied in the Takara kit and subjected to two rounds of PCR amplification with primers corresponding to the linker sequence and the primers Igfbp5-RP2 and Igfbp5-RP3. The amplified 3043 bp BglII–SalI fragment was cloned into the BamHI–SalI sites of the pBluescript SK+ (Stratagene) and partially sequenced. The Igfbp5 promoter fragment was excised by digesting at the flanking NotI (blunted) and XhoI sites, and ligated to the SmaI–XhoI sites of pGL3-basic (Promega) to generate pGL3 Igfbp5–2.9k. 5′ deletion constructs were generated by cloning shorter Igfbp5 promoter fragments digested with various restriction enzymes at the 5′ ends and with XhoI at the 3′ end into pGL3-basic. The following restriction sites were used in the present study: EcoRI (−1.4 kb, blunted), XbaI (−160, blunted), Eco130I (−105, blunted), SacI (−69) and HaeIII (−35).

Cells and transfection assays

P19 mouse embryonal carcinoma cells were grown in α-minimal essential medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (P/S) at 37°C under 5% CO_2. COS7 cells and human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and P/S at 37°C under 5% CO_2. For C2C12 mouse myoblasts, DMEM containing 4.5 g/l glucose was used. Mouse embryonic fibroblasts were prepared from 14.5 dpc embryos. Wild-type embryos were obtained from pregnant C57BL/6J-Jcl female mice purchased from CLEA Japan, Inc. (Tokyo). Six5-deficient embryos were obtained by crossing $Six5^{-/-}$; $Six4^{+/-}$ mice (15,37). Embryo genotyping was performed on DNA samples extracted from dissected head by PCR with the following primers: 5′-ACATCAAGCAGGAGAATGG-GATGG-3′ (downstream, the sequence present in both wild-type and mutagenized Six4 alleles), 5′-CCGTAATGGGA-TAGGTTACGTTGG-3′ (upstream, specific to mutagenized Six4 allele), and 5′-AGAAGTTCCGAGTGGAGTTGTACC-3′ (upstream, specific to wild-type Six4 allele). Cells at passages 5–7 were used in this study. Prior to RNA extraction, confluent cultures were serum-starved for 24 h in serum-free DMEM. Then, the cells were stimulated by replacing the serum-free medium with a fresh serum-free DMEM containing either 100 ng/ml IGF-II or 10% FCS and cultured for 12 h. Human fibroblast cultures were obtained from unaffected and DM1-affected adults as described previously (8). Normal (ST101) and DM1 fibroblasts (HFB-, HFC- and HFD-DM) were infected with pBABE MyoD-ER and pLXSN MyoD-ER, both of which expressed human MyoD fused to the ligand-binding domain of the estrogen receptor (ER) (73). Virus-infected fibroblasts were split in two, cultured to confluence and processed for RNA extraction before or after treatment with low-serum IT medium (DMEM, 2% heat-inactivated horse serum, 10 µg/ml transferrin, 10 µg/ml insulin) supplemented with 10^{-7} M β-estradiol for 72 h to convert to skeletal muscle.

Transfections were performed as described previously (19). Transfected cells were harvested after 48 h and assayed for luciferase and β-galactosidase activities. β-Galactosidase activity of the cotransfected reporter pEFBOSβ-gal was used as an internal control to correct for the variability in transfection efficiency.

Protein preparation and western blotting

Nuclear extracts were prepared from cultured cells as described previously (74). For virus-infected cells, 1 × Laemmli sample buffer was added to the culture dish, and the whole-cell lysate was used as a source of protein. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). VP16–Six5wt and VP16–Six5W241R expressed from plasmid or adenoviral vectors were detected using anti-VP16 (sc-7545 and sc-7546, Santa Cruz) and anti-Six5 (18) antibodies. For chemiluminescence detection, we used the ECL western blotting analysis system (Amersham Pharmacia Biotech).

Gel retardation assay

The gel retardation assay was carried out as described previously (75). Nuclear extracts prepared from transfected COS7 cells or GST–Six5, the SD and HD of Six5 fused to GST, purified from bacteria (13) were used in the assay. A BseDI(− 106)–BseDI(− 42) fragment of Igfbp5 promoter and the following double-stranded oligonucleotides were end-labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by Klenow fragment and used as probes: Igfbp5–105/79 (positions − 105 to − 79, 5′-CTTGGCATCCTTGCATGGG-TTGGGTGT-3′), Igfbp5–95/58 (positions − 95 to − 58, 5′-TGGGTGTTGGGGAGCTCAAATTGCAGCT-3′) and Igfbp5–64/39 (positions − 64 to − 39, 5′-TGCAGCTA-CAACTGGCTGGCAGCCAG-3′). C3WT (which corresponds to the Six4-binding site in Atpa1a promoter) and C3MUT were described previously (26). In some experiments, 20-fold molar excess of unlabeled oligonucleotides (C3WT and C3MUT) and anti-Six5 antiserum (0.2 µl) were added to the binding reaction.

Production and infection of recombinant adenovirus

We selected a replication-defective adenovirus vector AxCAwt (76), in which expression of a foreign gene is driven by a strong CAG promoter composed of the cytomegalovirus enhancer and chicken β-actin promoter. Recombinant adenoviruses were produced using the Adenovirus Expression Vector Kit (TaKaRa). DNA fragments encoding VP16–Six5wt and VP16–Six5W241R were excised from pCS2+ VP16 constructs by digesting at the flanking BamHI and SnaBI sites, blunted and cloned into the SwaI site of the cosmid vector pAxCAwt. The recombinant cosmids were cotransfected with the EcoT22I-digested AxCAwt DNA-terminal protein complex supplied in the Takara kit into 293 cells. Recombinant viruses were isolated, propagated and checked for any contaminating E1-bearing parent virus, titer [plaque forming unit (PFU)] and transgene expression. Virus titration was carried out using 293 cells and the expression of recombinant proteins was assessed by western blotting. For virus-infection experiments, cells plated onto a 10 cm culture dish were overlaid with recombinant adenoviruses [at a multiplicity of infection (m. o. i.) of 100–200 PFU] in culture medium containing 5% FCS for 2–3 h. The virus-containing medium was replaced with normal culture medium, and cells were further incubated for 6–48 h.

RNA extraction and northern blot analysis

Total RNA was extracted from cultured cells with Isogen reagent (Nippon Gene). Poly(A)$^+$ RNA was selected using Oligotex-dT30 'super' latex beads (TaKaRa). For northern blot analysis, total RNA was separated by electrophoresis through a 1.3% agarose/formaldehyde gel, blotted onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) and hybridized with ^{32}P-labeled cDNA probes. Igfbp5 and Igf2 cDNA fragments were isolated by PCR from a mouse skeletal muscle cDNA library (Clontech) using the following primers: Igfbp5-FP (5′-AGAATTCAATGGTGATCAGCG-TGGTCCTC-3′) and Igfbp5-RP (5′-AGATATCACTCAACGT-TACTGCTGTCG-3′) for the 831 bp Igfbp5 cDNA fragment and Igf2-FP (5′-AGAATTCAATGGGGATCCCAGTGGG-GAAG-3′) and Igf2-RP (5′-AGATATCACTGATGGTTGCTG-GACAT-3′) for the 558 bp Igf2 cDNA fragment, respectively. A 317 bp human IGFBP5 cDNA fragment was obtained by digesting the plasmid pHBP5–501 (77) with SacII(344) and SacI(660). Myogenin and β-actin cDNA fragments were prepared as described previously (37). Quantitation of the hybridization signals was carried out using the STORM system (Amersham Pharmacia Biotech) and ImageQuant software (Molecular Dynamics).

Expression profiling using a cDNA macroarray

Expression profiling was performed by hybridization of the Atlas Mouse 1.2 cDNA Expression Array containing 1176 known mouse cDNAs (Clontech) with ^{32}P-labeled cDNA probes. P19 cells infected with AxCAwt VP16–Six5wt and AxCAwt VP16–Six5W241R and cultured for 24 h were used as the source of poly(A)$^+$ RNA to prepare hybridization probes. The RNA samples were treated with RNase-free DNase I (Roche Diagnostics) before poly(A)$^+$ selection to remove any contaminating genomic DNA and subjected to PCR with the primers Six5–579F (5′-AGGATCCTGGCGAGGAGACCGTC-TA-3′) and Six5–820R (5′-TCTCGAGTGGACTCATC-CTCCGTAGTG-3′) to confirm that the samples produce no visible PCR products even after 35 cycles of amplification. Probe synthesis, hybridization and washing of the Atlas array were carried out as instructed by the manufacturer.

After a high-stringency wash instructed by the manufacturer, the hybridization pattern was quantified and analyzed using the STORM system and ArrayVision software (Imaging Research Inc.). The hybridization experiment was repeated using a set of RNAs prepared from P19 cells of a different passage number that were infected with the two recombinant adenoviruses. Genes whose expression levels were more than 2-fold higher in AxCAwt VP16–Six5wt-infected samples in both data sets were identified as differentially expressed genes.

DNase I footprinting and methylation interference assays

DNaseI footprinting and methylation interference assays were carried out as described previously (75). Purified GST–Six5, which comprises a region encompassing only the SD and HD (13), was used in the assays. The probe was prepared as follows. A BseDI(−106)–BseDI(−42) fragment of the Igfbp5 promoter was blunted and cloned into the SmaI site of pBluescript SK+. The insert was excised by digesting at the flanking SacII and HindIII sites. The HindIII site was 5′-labeled with [γ-^{32}P]dATP by T4 polynucleotide kinase or 3′-labeled with [α-^{32}P]dCTP by Klenow fragment.

ACKNOWLEDGEMENTS

We thank Keiko Ikeda for discussion and for critically reading the manuscript. We also thank Charles Thornton for discussion, Daniel Kessler for pCS2+ plasmids, Shunichi Shimasaki for pHBP5-501 and Hiroshi Yajima for technical advice on the production of recombinant adenovirus. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to K.K. and S.S.) and

the Cell Science Research Foundation (to S.S.) and the Research Grant (11B-1) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, Japan (to K.K.).

REFERENCES

1. Harper, P.S. (2001) Myotonic Dystrophy, 3rd edn. W.B. Saunders, London, UK.
2. Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.P., Hudson, T. et al. (1992) Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell, 68, 799–808.
3. Fu, Y.H., Pizzuti, A., Fenwick, R.G., King, J., Rajnarayan, S., Dunne, P.W., Dubel, J., Nasser, G.A., Ashizawa, T., de Jong, P. et al. (1992) An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science, 255, 1256–1258.
4. Mahadevan, M., Tsilfidis, C., Sabourin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barcelo, J., O'Hoy, K. et al. (1992) Myotonic dystrophy mutation: an unstable CTG repeat in the 3′ untranslated region of the gene. Science, 255, 1253–1255.
5. Fu, Y.H., Friedman, D.L., Richards, S., Pearlman, J.A., Gibbs, R.A., Pizzuti, A., Ashizawa, T., Perryman, M.B., Scarlato, G., Fenwick, R.G. et al. (1993) Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science, 260, 235–238.
6. Timchenko, L.T. (1999) Myotonic dystrophy: the role of RNA CUG triplet repeats. Am. J. Hum. Genet., 64, 360–364.
7. Murakami, Y., Ohto, H., Ikeda, U., Shimada, K., Momoi, T. and Kawakami, K. (1998) Promoter of mDMAHP/Six5: differential utilization of multiple transcription initiation sites and positive/negative regulatory elements. Hum. Mol. Genet., 7, 2103–2112.
8. Klesert, T.R., Otten, A.D., Bird, T.D. and Tapscott, S.J. (1997) Trinucleotide repeat expansion at the myotonic dystrophy locus reduces expression of DMAHP. Nature Genet., 16, 402–406.
9. Boucher, C.A., King, S.K., Carey, N., Krahe, R., Winchester, C.L., Rahman, S., Creavin, T., Meghji, P., Bailey, M.E., Chartier, F.L. et al. (1995) A novel homeodomain-encoding gene is associated with a large CpG island interrupted by the myotonic dystrophy unstable (CTG)n repeat. Hum. Mol. Genet., 4, 1919–1925.
10. Otten, A.D. and Tapscott, S.J. (1995) Triplet repeat expansion in myotonic dystrophy alters the adjacent chromatin structure. Proc. Natl Acad. Sci. USA, 92, 5465–5469.
11. Thornton, C.A., Wymer, J.P., Simmons, Z., McClain, C. and Moxley, R.T. (1997) Expansion of the myotonic dystrophy CTG repeat reduces expression of the flanking DMAHP gene. Nature Genet., 16, 407–409.
12. Kawakami, K., Sato, S., Ozaki, H. and Ikeda, K. (2000) Six family genes-structure and function as transcription factors and their roles in development. Bioessays, 22, 616–626.
13. Kawakami, K., Ohto, H., Takizawa, T. and Saito, T. (1996) Identification and expression of six family genes in mouse retina. FEBS Lett., 393, 259–263.
14. Heath, S.K., Carne, S., Hoyle, C., Johnson, K.J. and Wells, D.J. (1997) Characterisation of expression of mDMAHP, a homeodomain-encoding gene at the murine DM locus. Hum. Mol. Genet., 6, 651–657.
15. Klesert, T.R., Cho, D.H., Clark, J.I., Maylie, J., Adelman, J., Snider, L., Yuen, E.C., Soriano, P. and Tapscott, S.J. (2000) Mice deficient in Six5 develop cataracts: implications for myotonic dystrophy. Nature Genet., 25, 105–109.
16. Winchester, C.L., Ferrier, R.K., Sermoni, A., Clark, B.J. and Johnson, K.J. (1999) Characterization of the expression of DMPK and SIX5 in the human eye and implications for pathogenesis in myotonic dystrophy. Hum. Mol. Genet., 8, 481–492.
17. Sarkar, P.S., Appukuttan, B., Han, J., Ito, Y., Ai, C., Tsai, W., Chai, Y., Stout, J.T. and Reddy, S. (2000) Heterozygous loss of Six5 in mice is sufficient to cause ocular cataracts. Nature Genet., 25, 110–114.
18. Ohto, H., Takizawa, T., Saito, T., Kobayashi, M., Ikeda, K. and Kawakami, K. (1998) Tissue and developmental distribution of Six family gene products. Int. J. Dev. Biol., 42, 141–148.
19. Ohto, H., Kamada, S., Tago, K., Tominaga, S.I., Ozaki, H., Sato, S. and Kawakami, K. (1999) Cooperation of Six and Eya in activation of their target genes through nuclear translocation of Eya. Mol. Cell. Biol., 19, 6815–6824.
20. Spitz, F., Demignon, J., Porteu, A., Kahn, A., Concordet, J.P., Daegelen, D. and Maire, P. (1998) Expression of myogenin during embryogenesis is controlled by Six/sine oculis homeoproteins through a conserved MEF3 binding site. Proc. Natl Acad. Sci. USA, 95, 14220–14225.
21. Iannaccone, S.T., Bove, K.E., Vogler, C., Azzarelli, B. and Muller, J. (1986) Muscle maturation delay in infantile myotonic dystrophy. Arch. Pathol. Lab. Med., 110, 405–411.
22. Farkas-Bargeton, E., Barbet, J.P., Dancea, S., Wehrle, R., Checouri, A. and Dulac, O. (1988) Immaturity of muscle fibers in the congenital form of myotonic dystrophy: its consequences and its origin. J. Neurol. Sci., 83, 145–159.
23. Kirby, R.J., Hamilton, G.M., Finnegan, D.J., Johnson, K.J. and Jarman, A.P. (2001) Drosophila homolog of the myotonic dystrophy-associated gene, SIX5, is required for muscle and gonad development. Curr. Biol., 11, 1044–1049.
24. Mankodi, A., Logigian, E., Callahan, L., McClain, C., White, R., Henderson, D., Krym, M. and Thornton, C.A. (2000) Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Science, 289, 1769–1773.
25. Liquori, C.L., Ricker, K., Moseley, M.L., Jacobsen, J.F., Kress, W., Naylor, S.L., Day, J.W. and Ranum, L.P. (2001) Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. Science, 293, 864–867.
26. Kawakami, K., Ohto, H., Ikeda, K. and Roeder, R.G. (1996) Structure, function and expression of a murine homeobox protein AREC3, a homologue of Drosophila sine oculis gene product, and implication in development. Nucleic Acids Res., 24, 303–310.
27. Suzuki-Yagawa, Y., Kawakami, K. and Nagano, K. (1992) Housekeeping Na,K-ATPase alpha 1 subunit gene promoter is composed of multiple cis elements to which common and cell type-specific factors bind. Mol. Cell. Biol., 12, 4046–4055.
28. Harris, S.E., Winchester, C.L. and Johnson, K.J. (2000) Functional analysis of the homeodomain protein SIX5. Nucleic Acids Res., 28, 1871–1878.
29. Kobayashi, M., Nishikawa, K., Suzuki, T. and Yamamoto, M. (2001) The homeobox protein Six3 interacts with the Groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. Dev. Biol., 232, 315–326.
30. Liang, J., Moye-Rowley, S. and Maurer, R.A. (1995) In vivo mutational analysis of the DNA binding domain of the tissue-specific transcription factor, Pit-1. J. Biol. Chem., 270, 25520–25525.
31. Bain, G., Ray, W.J., Yao, M. and Gottlieb, D.I. (1994) From embryonal carcinoma cells to neurons: the P19 pathway. Bioessays, 16, 343–348.
32. Skerjanc, I.S. (1999) Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. Trends Cardiovasc. Med., 9, 139–143.
33. Oliver, G., Wehr, R., Jenkins, N.A., Copeland, N.G., Cheyette, B.N., Hartenstein, V., Zipursky, S.L. and Gruss, P. (1995) Homeobox genes and connective tissue patterning. Development, 121, 693–705.
34. Li, L., Cserjesi, P. and Olson, E.N. (1995) Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. Dev. Biol., 172, 280–292.
35. Saga, Y., Hata, N., Koseki, H. and Taketo, M.M. (1997) Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. Genes Dev., 11, 1827–1839.
36. Ema, M., Morita, M., Ikawa, S., Tanaka, M., Matsuda, Y., Gotoh, O., Saijoh, Y., Fujii, H., Hamada, H., Kikuchi, Y. et al. (1996) Two new members of the murine Sim gene family are transcriptional repressors and show different expression patterns during mouse embryogenesis. Mol. Cell. Biol., 16, 5865–5875.
37. Ozaki, H., Watanabe, Y., Takahashi, K., Kitamura, K., Tanaka, A., Urase, K., Momoi, T., Sudo, K., Sakagami, J., Asano, M. et al. (2001) Six4, a putative myogenin gene regulator, is not essential for mouse embryonal development. Mol. Cell. Biol., 21, 3343–3350.
38. Chen, C.M., Kraut, N., Groudine, M. and Weintraub, H. (1996) I-mf, a novel myogenic repressor, interacts with members of the MyoD family. Cell, 86, 731–741.
39. Green, B.N., Jones, S.B., Streck, R.D., Wood, T.L., Rotwein, P. and Pintar, J.E. (1994) Distinct expression patterns of insulin-like growth factor binding proteins 2 and 5 during fetal and postnatal development. Endocrinology, 134, 954–962.
40. Lee, J.E., Pintar, J. and Efstratiadis, A. (1990) Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. Development, 110, 151–159.

41. Melkonyan, H.S., Chang, W.C., Shapiro, J.P., Mahadevappa, M., Fitzpatrick, P.A., Kiefer, M.C., Tomei, L.D. and Umansky, S.R. (1997) SARPs: a family of secreted apoptosis-related proteins. Proc. Natl Acad. Sci. USA, 94, 13636–13641.
42. Borello, U., Buffa, V., Sonnino, C., Melchionna, R., Vivarelli, E. and Cossu, G. (1999) Differential expression of the Wnt putative receptors Frizzled during mouse somitogenesis. Mech. Dev., 89, 173–177.
43. Garel, S., Marin, F., Mattei, M.G., Vesque, C., Vincent, A. and Charnay, P. (1997) Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. Dev. Dyn., 210, 191–205.
44. Kaestner, K.H., Monaghan, A.P., Kern, H., Ang, S.L., Weitz, S., Lichter, P. and Schutz, G. (1995) The mouse fkh-2 gene. Implications for notochord, foregut, and midbrain regionalization. J. Biol. Chem., 270, 30029–30035.
45. Parr, B.A., Shea, M.J., Vassileva, G. and McMahon, A.P. (1993) Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development, 119, 247–261.
46. Jursky, F. and Nelson, N. (1999) Developmental expression of the neurotransmitter transporter GAT3. J. Neurosci. Res., 55, 394–399.
47. Maricq, A.V., Peterson, A.S., Brake, A.J., Myers, R.M. and Julius, D. (1991) Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel. Science, 254, 432–437.
48. Jones, J.I. and Clemmons, D.R. (1995) Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev., 16, 3–34.
49. Stylianopoulou, F., Efstratiadis, A., Herbert, J. and Pintar, J. (1988) Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. Development, 103, 497–506.
50. Stylianopoulou, F., Herbert, J., Soares, M.B. and Efstratiadis, A. (1988) Expression of the insulin-like growth factor II gene in the choroid plexus and the leptomeninges of the adult rat central nervous system. Proc. Natl Acad. Sci. USA, 85, 141–145.
51. Duan, C. and Clemmons, D.R. (1995) Transcription factor AP-2 regulates human insulin-like growth factor binding protein-5 gene expression. J. Biol. Chem., 270, 24844–24851.
52. Yeh, L.C. and Lee, J.C. (2000) Identification of an osteogenic protein-1 (bone morphogenetic protein- 7)-responsive element in the promoter of the rat insulin-like growth factor-binding protein-5 gene. Endocrinology, 141, 3278–3286.
53. Boonyaratanakornkit, V., Strong, D.D., Mohan, S., Baylink, D.J., Beck, C.A. and Linkhart, T.A. (1999) Progesterone stimulation of human insulin-like growth factor-binding protein-5 gene transcription in human osteoblasts is mediated by a CACCC sequence in the proximal promoter. J. Biol. Chem., 274, 26431–26438.
54. Duan, C., Liimatta, M.B. and Bottum, O.L. (1999) Insulin-like growth factor (IGF)-I regulates IGF-binding protein-5 gene expression through the phosphatidylinositol 3-kinase, protein kinase B/Akt, and p70 S6 kinase signaling pathway. J. Biol. Chem., 274, 37147–37153.
55. Kou, K., Mittanck, D.W., Fu, C. and Rotwein, P. (1995) Structure and function of the mouse insulin-like growth factor binding protein 5 gene promoter. DNA Cell Biol., 14, 241–249.
56. Parmacek, M.S., Ip, H.S., Jung, F., Shen, T., Martin, J.F., Vora, A.J., Olson, E.N. and Leiden, J.M. (1994) A novel myogenic regulatory circuit controls slow/cardiac troponin C gene transcription in skeletal muscle. Mol. Cell. Biol., 14, 1870–1885.
57. Duan, C., Hawes, S.B., Prevette, T. and Clemmons, D.R. (1996) Insulin-like growth factor-I (IGF-I) regulates IGF-binding protein-5 synthesis through transcriptional activation of the gene in aortic smooth muscle cells. J. Biol. Chem., 271, 4280–4288.
58. Smink, J.J., Koster, J.G., Hendriks-Stegeman, B.I. and Van Buul-Offers, S.C. (1999) Insulin-like growth factor (IGF) II induced changes in expression of IGF binding proteins in lymphoid tissues of hIGF-II transgenic mice. Endocrinology, 140, 5876–5882.
59. Holthuizen, P.E., Cleutjens, C.B., Veenstra, G.J., van der Lee, F.M., Koonen-Reemst, A.M. and Sussenbach, J.S. (1993) Differential expression of the human, mouse and rat IGF-II genes. Regul. Pept., 48, 77–89.
60. Ainscough, J.F., John, R.M., Barton, S.C. and Surani, M.A. (2000) A skeletal muscle-specific mouse Igf2 repressor lies 40 kb downstream of the gene. Development, 127, 3923–3930.
61. DeChiara, T.M., Efstratiadis, A. and Robertson, E.J. (1990) A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature, 345, 78–80.
62. Khan, J., Bittner, M.L., Saal, L.H., Teichmann, U., Azorsa, D.O., Gooden, G.C., Pavan, W.J., Trent, J.M. and Meltzer, P.S. (1999) cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. Proc. Natl Acad. Sci. USA, 96, 13264–13269.
63. Savkur, R.S., Philips, A.V. and Cooper, T.A. (2001) Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nature Genet., 29, 40–47.
64. Furling, D., Marette, A. and Puymirat, J. (1999) Insulin-like growth factor I circumvents defective insulin action in human myotonic dystrophy skeletal muscle cells. Endocrinology, 140, 4244–4250.
65. Vlachopapadopoulou, E., Zachwieja, J.J., Gertner, J.M., Manzione, D., Bier, D.M., Matthews, D.E. and Slonim, A.E. (1995) Metabolic and clinical response to recombinant human insulin-like growth factor I in myotonic dystrophy-a clinical research center study. J. Clin. Endocrinol. Metab., 80, 3715–3723.
66. Louvi, A., Accili, D. and Efstratiadis, A. (1997) Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev. Biol., 189, 33–48.
67. Burren, C.P., Berka, J.L. and Batch, J.A. (1997) Localization studies of IGFBP-2 and IGFBP-5 in the anterior compartment of the eye. Curr. Eye Res., 16, 256–262.
68. Danias, J. and Stylianopoulou, F. (1990) Expression of IGF-I and IGF-II genes in the adult rat eye. Curr. Eye Res., 9, 379–386.
69. Civil, A., van Genesen, S.T., Klok, E.J. and Lubsen, N.H. (2000) Insulin and IGF-I affect the protein composition of the lens fibre cell with possible consequences for cataract. Exp. Eye. Res., 70, 785–794.
70. Shirke, S., Faber, S.C., Hallem, E., Makarenkova, H.P., Robinson, M.L., Overbeek, P.A. and Lang, R.A. (2001) Misexpression of IGF-I in the mouse lens expands the transitional zone and perturbs lens polarization. Mech. Dev., 101, 167–174.
71. Ozaki, H., Watanabe, Y., Ikeda, K. and Kawakami, K. (2002) Impaired interactions between Eya1 harboring mutations found in patients with branchio-oto-renal syndrome and Six, Dach and G proteins. J. Hum. Genet., 47, 107–116.
72. Kessler, D.S. (1997) Siamois is required for formation of Spemann's organizer. Proc. Natl Acad. Sci. USA, 94, 13017–13022.
73. Hollenberg, S.M., Cheng, P.F. and Weintraub, H. (1993) Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. Proc. Natl Acad. Sci. USA, 90, 8028–8032.
74. Kawakami, K., Yanagisawa, K., Watanabe, Y., Tominaga, S. and Nagano, K. (1993) Different factors bind to the regulatory region of the Na^+, K^+-ATPase alpha 1-subunit gene during the cell cycle. FEBS Lett., 335, 251–254.
75. Kawakami, K., Scheidereit, C. and Roeder, R.G. (1988) Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-kappa B) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc. Natl Acad. Sci. USA, 85, 4700–4704.
76. Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. and Saito, I. (1995) Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res., 23, 3816–3821.
77. Shimasaki, S., Shimonaka, M., Zhang, H.P. and Ling, N. (1991) Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. J. Biol. Chem., 266, 10646–10653.